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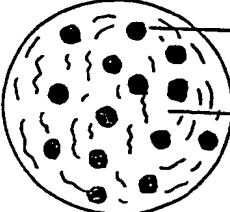
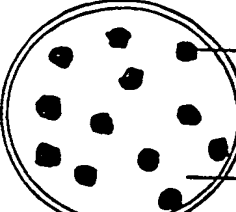
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<p>(21) International Application Number: PCT/US90/04381 (22) International Filing Date: 6 August 1990 (06.08.90) (30) Priority data: 389,455 7 August 1989 (07.08.89) US (71)(72) Applicants and Inventors: SCHLAMEUS, Herman, Wade [US/US]; 7106 Quail Garden, San Antonio, TX 78250 (US). FOX, William, Casey [US/US]; 7418 Pebblewood, San Antonio, TX 78250 (US). MANGOLD, Donald, Jacob [US/US]; 14711 Bold Venture, San Antonio, TX 78248 (US). TRIPLETT, Robert, Gill [US/US]; 2906 Bee Cave, San Antonio, TX 78231 (US). HOLT, George, Richard [US/US]; 100 Pin Oak Forest, San Antonio, TX 78232 (US). AUFDEMORTE, Thomas, Bruce [US/US]; 1638 Vista Del Monte, San Antonio, TX 78219 (US).</p>		<p>(74) Agent: GOODMAN, Rosanne; Fulbright & Jaworski, 300 Convent Street, Suite 2200, San Antonio, TX 78205 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: COMPOSITION AND METHOD OF PROMOTING HARD TISSUE HEALING</p> <p>(57) Abstract</p> <p>Osteoprogenitor cells encapsulated in alginate and alternatively, additionally encapsulated in polylysine and/or agarose promote regeneration of bone at the site of implantation. The present invention provides a composition comprising osteoprogenitor cells embedded or encapsulated in alginate and the use of said microcapsules for the facilitation of bone regeneration.</p> <div style="display: flex; justify-content: space-around; align-items: center;">  <div style="text-align: right;"> <p>Live Cells</p> <p>Algln Matrix</p> </div> </div> <div style="display: flex; justify-content: space-around; align-items: center; margin-top: 20px;">  <div style="text-align: right;"> <p>Live Cells</p> <p>Polylysine-Algln Membrane</p> <p>Liquid Medium</p> </div> </div> <p style="text-align: center; margin-top: 20px;">TWO TYPES OF ARTIFICIAL CELLS</p>		

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-1-

COMPOSITION AND METHOD OF PROMOTING
HARD TISSUE HEALING

15 Field of the Invention

The present invention relates generally to the field of hard tissue healing and, more particularly, to the field of biodegradable implantable microcapsules to stimulate the natural process of hard tissue regeneration and bone wound healing.

Background of the Invention

Defects in bone or osseous structures will initiate the process of bone healing. Healing often involves the replacement of injured tissue by connective tissue and leaves a scar. Bone, under optimal conditions, heals by regeneration in which injured tissues are replaced by their own kind and leave no scar. The success of regeneration following injury depends, among other things, on the type of injury, the adequacy of treatment and the systemic health of the patient. Osseous repair involves at least six physiological stages: impact, induction, inflammation, soft callus formation, hard

35

-2-

1 callus formation, remodeling and regeneration.
Heppenstall, Fracture Treatment and Healing, W.B.
Saunders, Philadelphia, 1980, page 35.

5 With inadequate treatment, severe injury and/or
metabolic bone disease, fracture healing is significantly
retarded. For example, in the case of a metabolic bone
disease such as osteoporosis, 40% of patients with
decreased bone mass due to osteoporosis showed a markedly
impaired fracture repair rate. Only 33% of women in whom
10 significant osteoporosis was present were able to achieve
a solid union following femoral neck fractures. In
comparison, in 90% of women with physiologically normal
bone mass a successful union was achieved. Lane et al.,
Osteoporosis, Orthopedics clinics North America 15: 711
15 (1984); Arnold, J. Bone Joint Surg. 66A: 847 (1984);
Scileppe et al., Surg. Form 32: 543 (1981).

It is estimated that there are 200,000 hip
fractures in osteoporetic women in the United States
annually with a 40% mortality rate due to complications of
20 repair of these fractures. As a result, there is a
significant need to facilitate fracture repair in these
types of patients. In addition, fractures in young
accident and trauma victims result in loss of numerous
productive days from the work place. For example, it
25 takes an average of six weeks to complete repair even
simple bone injuries in healthy individuals.

Bone fractures and bone wound healing following
trauma or surgery account for considerable morbidity and
mortality. For example, femoral neck fractures in
30 patients under forty may be associated with avascular
necrosis in as many as 40% of cases complicated by
non-union. Kyle et al., Young Femoral Neck Fractures,
Presented at the 52st Annual Meeting of American Acadeym
of Orthopedic Surgery, Atlanta, Ga. (1984). Many other
35 examples could be cited of the need for more expeditious

-3-

1 methods to facilitate and/or accelerated fracture or hard
tissue defect repair.

In addition, recent technological advances have
made the replacement of joints and defective or diseased
5 hard tissues common surgical procedures.

Since the feasibility of the preparation of
artificial cells was first demonstrated in 1957 by Chang
(Chang, T.M.S. (1964) Science:146, 524), numerous
approaches to their production and use have been
10 evaluated. Artificial cell membranes have been reported
using a variety of synthetic and biological materials to
give the desired membrane properties. A large variety of
materials can be enclosed (microencapsulated) in
artificial cells. This includes single and multienzyme
15 systems, cell extracts, and combined enzyme-adsorbent
systems (Chang, U.S. Patent No. 4,642,120). Biological
cells have been encapsulated to prevent them from being
adversely affected by external factors and immunological
rejection (Chang, Biomedical Applications of Immobilized
20 Enzymes and Proteins (Plenum: New York, 1977) Vols. 1 and
2; Mosbach et al. (1966) Acta Chem. Scan. 20: 2807; Lim et
al. (1980) Science 210:908). More recently the
microencapsulation of living biological cells that can be
maintained in culture has been disclosed (Lim et al.
25 (1980) Science 210: 908; U.S. Patent No. 4,391,909).

U.S. Patent No. 4,663,286 (Tsang et al.)
discloses a process for encapsulating material is
described for forming a capsule utilizing an alginate
polymer with a polyvalent cation.

30 U.S. Patent No. 4,642,120 (Nevo et al.) discloses
the repair of cartilage and bones by employment of a
composition provided in gel form. The gel comprises
certain types of cells. These may be committed embryonal
chondrocytes or any type of mesenchymally-derived cells
35 which may differentiate into chondrocytes, generally as a

1 consequence of the influence of chondrogenic inducing
factors, in combination with fibrinogen, antiprotease,
thrombin, and other factors. According to U.S. Patent No.
4,642,120, the cells should be of the same species as that
5 to which the composition is transplanted. Incorporation
of extracellular matrix (ECM) of chondrocytes, other
hormones and/or growth factors such as SM (Somatomedin or
IGF-I), FGF (fibroblast growth factor), CGF (cartilage
growth factor), BDGF (bone derived growth factor) or a
10 combination of any of these in the gel is also disclosed.

U.S. Patent No. 4,472,840 (Jefferies) discloses a
method of inducing osseous formation by implanting bone
graft material. Both demineralized bone particles (DBP)
and bone inductive protein have demonstrated the capacity
15 to induce the formation of osseous tissue in animal and
human experiments. Reconstituted collagen conjugate is
known to be highly biocompatible and can be fabricated in
a variety of configurations, especially as a sponge. This
material can be used as a grafting implant in plastic and
20 reconstructive surgery, periodontal bone grafting, and in
endodontic procedures. Structural durability is enhanced
by crosslinking with glutaraldehyde which is also used to
sterilize and disinfect the collagen conjugate prior to
implantation.

25 U.S. Patent No. 4,132,746 (Urry et al.) discloses
a crosslinked insoluble polypentapeptide elastomer capable
of calcification by withdrawing calcium ions from a serum
medium, thus making it useful as a calcifiable matrix for
the formation of an artificial bone structure. The
30 calcifiable material can be treated to make it useful in
artificial vascular wall formation.

U.S. Patent No. 4,609,551 (Caplan et al.)
discloses a material for stimulating growth of cartilage
and bony tissue at anatomical sites. The material
35 consists of a composition with a fibrin or allograft

-5-

1 matrix containing soluble bone protein and fibroblast
cells.

Summary of the Invention

5 In order to facilitate the healing of bone and
other hard tissue fractures and defects and facilitate
structural implant fixation, a microcapsule has been
developed. Specifically, it has been discovered that
10 osteoprogenitor cells can be embedded in or encapsulated
by biocompatible materials and nonetheless retain their
viability and biological function. The biocompatible
encapsulating materials useful in practicing this
invention can have different rates of biodegradability.
The biocompatible material may be readily biodegradable,
15 slowly biodegradable or relatively resistant to
degradation in biological fluids. A readily
biodegradable material is one that is degraded 50% or
more within hours to several days by contact with
biological fluids. A slowly biodegradable material will
20 degrade at least 50% when in contact with biological
fluids for more than several days up to a week or several
weeks. A material resistant to biodegradation is one
which retains its integrity for at least several weeks in
the presence of biological fluids.

Materials which are readily biodegradable
25 (bioerodable) include naturally-occurring polymers such as
alginates, polylysine, cellulose polymers, e.g.,
methylcellulose, collagen, gellan gum, casein, chitosan,
and the like. Materials which are slowly degradable
include some polyesters, and polyanhydrides.
30 Biocompatible materials which are relatively resistant to
biodegradation include titanium oxide, hydroxyapatite,
biocompatible metal compositions, biocompatible ceramic
compositions, and the like. The microcapsules of the
present invention can comprise one biodegradable material
35 or a combination of two or more biodegradable materials.

-6-

1 In the latter case the microcapsule may contain
biocompatible materials of varying rates of
biodegradability.

5 A microcapsule comprising one or more
biodegradable materials can itself be coated or further
encapsulated by a less readily degradable substance in
order to further delay complete release of the
encapsulated material. By carefully choosing the
10 materials used as the initial encapsulating material and
the subsequent coating or encapsulating material, one of
skill in the art may control the rate of release of one or
several encapsulated materials, including the encapsulated
osteoprogenitor cells. For instance, in one embodiment,
alginate alone can be used as the sole encapsulating
15 material. In a second embodiment, biodegradability is
retarded by coating thus-prepared alginate microcapsules
with a polyanionic polymer such as polylysine.

In yet another embodiment a core material
relatively resistant to biodegradation, such as a ceramic
20 material to which another material, e.g., one of the above
mentioned growth factors, has been bound and from which
this other material is slowly released, e.g., from the
surface of the core material, may be encapsulated within a
more readily biodegradable material which itself contains
25 the same or other treating materials, e.g., the same or
another growth factor, antiviral agent, hormone, in order
to sustain release of one or more of the encapsulated
materials. For instance, a microcapsule comprising woven
titanium mesh mixed with collagen may be also be embedded
30 within the algin microcapsule containing osteoprogenitor
cells. Prosthetic devices formed of the present invention
will facilitate fixation of orthopedic devices or dental
implants by enhancing the bone regeneration at the site of
prosthetic implantation. In another embodiment, fixation
35 of orthopedic implants at the surgical site can be

-7-

1 facilitated by implantation of the composition of the
present invention comprising ceramic hydroxyapatite
adsorbed with bone derived growth factor or any other
5 material which stimulates the differentiation or growth of
osteoprogenitor or cartilage progenitor cells with the
progenitor cells in the formable materials useful for
practicing the present invention. This embodiment allows
a stable and solid support for replacement and/or
10 reconstruction of defective hard tissues while
additionally providing the necessary progenitor cells to
repair and/or replace the defective hard tissue
structures.

As indicated above, microcapsules prepared in
accordance with this invention can additionally contain
15 materials which aid in bone healing or in the prevention
or treatment of complications of trauma. Such additional
materials can include, but are not limited to,
extracellular matrix of chondrocytes (ECM), hormones,
growth factors such as somatomedins, fibroblast growth
20 factor, bone morphogenic protein, platelet derived growth
factor, bone inductive growth factor, osteoinductive
growth factor, cartilage derived growth factor,
prostaglandins, macrophage derived growth factors, bone
derived growth factor, skeletal derived growth factor,
25 epidermal growth factor, transforming growth factor β ,
growth factor, cytokines, and the like, or a combination
of any of these. Such materials may alternatively be
termed herein hard tissue promoting factors. Other agents
which aid in treatment or prevention of the complications
30 of trauma may additionally be included. Examples of such
other agents are, without limitation, antiviral agents,
antibacterial agents and the like. The above agents and
factors may be used alone or in combination in practicing
the present invention. Such materials can be prepared by
35 any method known to those skilled in the art, including

-8-

1 purification from naturally occurring sources and
recombinant technology.

5 The microcapsules of this invention, coated or
uncoated, e.g., with polylysine, can be further surrounded
by a material that can be formed into a hydrogel wafer,
such as agar, gelatin, gellan gum or the like, in order to
facilitate handling and transfer or implantation of
encapsulated material(s) into the site of treatment.

10 The present invention provides compositions and a
method to facilitate the healing or regeneration of bone,
for instance, at fracture sites. This method comprises
implantation or injection of any of the compositions of
the present invention into a site or a device in an
individual at which bone fixation, reconstruction,
15 regeneration or healing is desired. The osteoprogenitor
cells then proliferate and cause the deposition of new
bone material at the implantation or injection site.

The present invention also provides compositions
and a method to facilitate the regeneration and healing of
20 cartilagenous tissues.

It is, therefore, an object of the present
invention to provide a composition to augment and/or
facilitate the regeneration or healing of bone tissue at
fracture sites.

25 A further object of the present invention is to
provide a wafer delivery system for encapsulated
osteoprogenitor cell-containing compositions of the
present invention.

30 Yet another objective of the present invention is
to provide viable encapsulated osteoprogenitor cells for
implantation and timed-release at bone fracture sites to
augment and facilitate healing of the fracture.

A still further object of the present invention
is to provide a method of stimulating the healing of bone
35 fractures.

-9-

1 These and other objects, as well as the nature,
scope, advantages and utilization of this invention, will
become readily apparent to those skilled in the art from
the following description, the drawings and the appended
5 claims.

Brief Description of the Drawings

Figure 1 shows a schematic of the two capsule
types.

10 Figure 2 shows a schematic of the type of
apparatus used to form one type of microcapsule.

Detailed Description of the Invention

15 In order to accomplish the above objects and
objectives, the present invention provides, in one
embodiment, osteoprogenitor cells embedded or encapsulated
in an alginate matrix.

20 In one embodiment of the present invention,
osteoprogenitor cells have been encapsulated, viability
maintained within artificial membranes, and the cells when
implanted in an animal model, subsequently proliferate and
maintain their capacity to induce osteogenesis.

25 The osteoprogenitor cells useful in carrying out
the present invention can be any cells capable of inducing
the formation of regenerated bone (or cartilage)(or the
deposition of calcium?). Preferably, these cells are
autologous bone progenitor cells harvested from the
individual in need of such treatment. These cells may be
harvested from the site of the injury or from a distant
site for transplantation to the injury site. The primary
cells may be used directly or may be expanded by passage
30 in cell culture. In another embodiment, the
osteoprogenitor cells may be harvested from another
individual of the same specie as the individual to be
treated. However, the cells may also be selected from
the group consisting of cell lines derived from any
35 mesenchymally cells which will differentiate to form

-10-

1 osseous or cartilaginous tissue. In selecting the
osteoprogenitor cells for use in the present invention it
is only important to try to minimize as much as possible
the rejection of the implanted cells for the period
5 necessary to induce the regeneration of new bone.

The preferred composition of the present
invention comprises osteoprogenitor cells embedded or
encapsulated in a biodegradable material. The cells may
either be embedded in a matrix material by being dispersed
10 within the matrix material itself or by surrounding the
cells with a biodegradable material. In either case, in
order to decrease the rate of release of the
osteoprogenitor cells from the microcapsule, the cells may
be further encapsulated in a nonbiodegradable material or
15 a material which has a prolonged integrity in the host
such as polylysine. Preferably, the matrix material is an
alginate, such as sodium alginate. The matrix material
may also be selected from the group consisting of gellan
gum, chitosan, or agarose.

20 The method of encapsulating the osteoprogenitor
cells comprises embedding or encapsulating the cells in a
biodegradable material by any of the techniques known to
those of skill in the art. Preferably the osteoprogenitor
cells are encapsulated by a modification of the method
25 disclosed in U.S. Patent No. 4,391,909, incorporated
herein by reference. Briefly, osteoprogenitor cells were
gently dispersed in a solution of sterile sodium alginate
and pumped through a needle into a collection bath of 1.3%
calcium chloride containing Tween 20. The alginate
30 embedded cells, also termed herein microcapsules, were
harvested, washed with saline and either used directly for
implantation or injection into the treatment site or
further encapsulated to prolong the integrity in the host.

In a preferred embodiment, the microcapsules were
35 formed into wafers to facilitate implantation. These

-11-

1 wafers were preferably composed of agar, such a wafer is
described in Example 4. The wafer can be of any material
that is biocompatible and can be formed into a hydrogel
5 having characteristics similar to agar such that the
handling and placement of the microcapsules at the
treatment site is facilitated.

The method of treating bone fractures of the
present invention comprises implantation of the
osteoprogenitor microcapsules of the present invention
10 into a fracture site of an individual and allowing
sufficient time for the formation of new bone at the
treatment site. The osteoprogenitor microcapsules may be
implanted by surgical procedures known to those of skill
in the art or may be injected into the fracture site
15 utilizing a suitable pharmaceutical carrier. The choice
of such carriers will be obvious to those in the art.

The term "individual" is meant to include any
animal, preferably a mammal, and most preferably a human,
cat, dog, or horse.

20 Artificial cell preparation was carried out in a
sterile environment. All equipment, materials, solutions,
etc. were either sterilized by the appropriate means or
purchased as sterile before use in the process.

Having now generally described the invention, a
25 more complete understanding can be obtained by reference
to the following specific examples. These examples are
provided for purposes of illustration only and are not
intended to be limiting unless otherwise specified.

30 Example 1

PROCEDURE FOR ENCAPSULATION OF OSTEOPROGENITOR CELLS

A. Preparation of osteoprogenitor cells.

Cells were isolated from canine trabecular bone
specimens. The specimens represented material obtained by
35

-12-

1 biopsy of the iliac crest of four research grade mongrel
dogs numbered as follows: 4452, 4386, 4593, and 4467.
The biopsy specimens from each dog were processed
5 individually in order to permit autologous implantation of
the cellular material at a later date, thereby
circumventing any possible rejection response and
eliminating the need for immune suppression of the host
dogs.

The biopsy material was washed multiple times in
10 Dulbecco's modified Eagle's medium (DMEM) containing
penicillin (1000 units/ml), streptomycin (1000 ug/ml), and
amphotericin-B (0.25 ug/ml) to remove adherent tissue and
debris. The bony trabeculae were then cut into small
pieces (1-2 mm²) followed by a second series of washings
15 to remove hematogenous elements. The resulting clean
pieces of bone were placed in a 100 mm cell culture dish
in the absence of media and incubated at 37°C in an
atmosphere of O₂/CO₂ (95/5 v/v). After 20 minutes, 10
ml DMEM containing 10% newborn calf serum (NCS) was
20 carefully added to the dish without disturbing the bone
fragments. The dishes were returned to the incubator and
left undisturbed for 5 days. Subsequently, the media was
changed every three days to fresh DMEM, 10% NCS. After 23
days of culture, the cells which had migrated from the
25 bone fragments onto the surface of the culture dish were
removed with trypsin/EDTA (0.125%/1 mM).

These cells were placed in a T-75 culture flask
and designated first passage cells. The cells were
passaged two more times to yield third passage cells
30 which, when confluent, were encapsulated as described
below (Runs 1-30A through 1-31B). Examination of aliquots
of the encapsulated cells suspended in DMEM containing the
vital dye trypan blue, indicated that the cells had
retained their viability during the encapsulation.

35

-13-

1 procedure. The encapsulated cells were maintained in DMEM
containing 10% NCS at 37°C in an atmosphere of O_2/CO_2
(95/5 v/v) for 24-48 hours prior to preparation for
5 implantation into nonunion sites prepared in the radii of
dogs. Viability experiments revealed that the
encapsulated cells could be maintained in this manner for
three days without a decrease in cell number. In fact,
the cell number increased by 70-90% during this time
period.

10 Cells for implantation in nonunion fracture sites
in dogs were harvested and grown in culture as described
above. Osteoprogenitor cells were incubated in an
incubator (37°C) until ready for use in the encapsulation
process.

15 B. Encapsulation of Cells

Cells were encapsulated by a modification of the
method described in U.S. Patent No. 4,391,909 (Lim). Two
types of encapsulated cells were prepared. In one, cells
were encapsulated (or embedded) in an algin matrix. In
20 the second, the process was carried further and the
alginate embedded cells were further encapsulated using
poly-L-lysine/alginate as the capsule membrane. A schematic
of the two capsule types is shown in Figure 1.

The encapsulated cells were prepared as follows
25 and used for implantation into animals to demonstrate the
effect on fracture healing. Cells from several flasks
were combined, placed in a 15-ml sterile culture tube and
rinsed 3 times with sterile 0.9% saline solution. After
decanting the saline solution from culture tube, 10 ml of
30 sterile sodium alginate solution (about 1%) was added.
The alginate used for most of the cell encapsulation was
sterile Macrocarrier* solution obtained from Bellco Glass,
Inc. The cells were gently dispersed and the
cell/alginate solution was transferred to a sterile
35 syringe. The syringe was placed in a sterile pump device

-14-

1 and connected to the encapsulation device with sterile
tubing.

5 A sterile collection bath (containing a 1.3%
calcium chloride solution with 0.25 ml of 10% Tween 20)
was placed under the encapsulation device. The cells were
encapsulated in the alginate and collected in the
collection bath. After the alginate encapsulated cells
remained in the collection bath for 3-5 minutes, they were
passed through fine wire screen baskets. The alginate
10 embedded (encapsulated) cells were then rinsed 2 times
with 0.9% saline solution and used in this form as the
alginate matrix cell preparation.

In some situations when it is desirable to
provide polylysine encapsulated osteoprogenitor cells, the
15 alginate embedded (encapsulated) cells were rinsed once
with a polylysine solution, preferably about 0.2%. The
poly-L-lysine used in the encapsulation was obtained from
Sigma Chemical Company and had a molecular weight of
approximately 38,000. The cells were then incubated in
20 the polylysine solution for 5-7 minutes, rinsed 2 times
with 0.9% saline solution, and finally, rinsed once with
an approximately 1.5% sodium citrate solution by
incubating the encapsulated cells in the sodium citrate
solution for 5-7 minutes. The cells were then rinsed 2
25 times with 0.9% saline solution and 3 times with DME
(Dulbecco's Modified Eagle's Medium) for 2-3 minutes.

The cells suspended in approximately 40 ml DME
were transferred to a sterile T-75 flask and incubated at
37°C until implantation.

30 The results of the encapsulation procedures are
shown on Table 1. In the initial runs (1-1A through 1-9D)
only placebo capsules were prepared in order to adjust
process parameters to produce the desired type of
capsule. Matrix materials evaluated during this period
35 included alginates, casein, chondroitin sulfate, and

-15-

1 collagen. In the preferred embodiment, spheres were
formed using sodium alginate collected in a calcium
chloride (CaCl₂) bath as shown in Run 1-7A in Table 1. In
5 forming the capsule, air regulation was used to control
the droplet size. A schematic of the apparatus used is
shown in Figure 2.

Runs following 1-12A were carried out with
encapsulating live cells unless otherwise stated. The
encapsulation of osteoprogenitor cells are designated as
10 1-30A through 1-31B in Table 1.

Histopathologic analysis was performed on
encapsulated cells maintained in vitro as well as on
tissues removed at necropsy from animals implanted with
microencapsulated osteoprogenitor cells for in vivo
15 evaluation. This was accomplished in three phases as
described in Examples 2, 3, and 4, below.

Example 2

In Vitro Cell Analysis

20 Following encapsulation of the cells, in vitro
studies were conducted to determine osteoprogenitor cell
viability and define their morphology within artificial
cell membranes. Histologic sections were prepared and
stained with hematoxylin and eosin using encapsulated
25 cells in the following combinations:

- K-1 Alginate + U2OS cells (an osteosarcoma cell line)
- K-2 Polylysine + U2OS cells
- K-3 Alginate + normal dog cells (animal #4452)
- 30 K-4 Polylysine + normal dog cells (animal #4452)
- K-5 Alginate + FL cell tumor (a transformed human
tumor cell line capable of bone formation)

U2OS cells encapsulated in alginate appeared as
small nests or colonies numbering approximately 2-15
35 cells, each with an average of approximately 10 cells per

-16-

1 group. The cells had basophilic staining nuclei which
were round and regular with prominent nucleoli noted at
random. Cell cytoplasm was moderately eosinophilic and
cell boundaries were relatively distinct. The algin
5 matrix was amorphous and slightly basophilic but obviously
degrading as a consequence of the histologic processing
procedure necessary to produce the sections.

U2OS cells encapsulated in polylysine also
appeared as clusters with morphology not significantly
10 different from that described above, however, the
artificial polylysine membranes were histologically
distinct as slightly basophilic undulating cuticular
surfaces enclosing cell nests. The undulation was
interpreted as an artifact of dehydration, again necessary
15 for processing.

Normal dog cells when encapsulated in alginate,
appeared as isolated groups, usually of 2-3 cells.
Morphologically the cells had the characteristics of
osteoblasts with eccentrically located round nuclei and
20 relatively conspicuous eosinophilic cytoplasm. In some
cells there was evidence of a perinuclear eosinophilic
condensation typical of osteoblasts. Again, the alginate
membranes appeared to be degrading as a result of the
histological preparation.

25 Normal osteoprogenitor dog cells encapsulated in
polylysine showed similar morphology to those encapsulated
in alginate alone. Again, polylysine membranes were
distinct as described with the U2OS cells above.

Alginate embedded FL cells also showed isolated
30 cells or groups of 2-4 cells with round, eccentrically
located nuclei, occasional prominent nucleoli and
eosinophilic cytoplasm, but differing from U2OS cell lines
in that clusters were in general smaller and less numerous
within the artificial membranes.

35

-17-

1 In summary, all artificial cell preparations
contained viable cells with morphology varying as to the
derivation of the particular cell type indicating that no
deleterious effects resulted from the encapsulation
5 process.

 Alginate cell membranes degraded during
histological processing and thus were not visible in
subsequent sections produced from animal studies.
Polylysine membranes were more distinct and durable and
10 remained visible at least in early phases of the animal
studies. The interpretation of the in vivo data shown in
the Examples below was made in accordance with these
observations .

 These studies demonstrate that cells may be
15 encapsulated, their viability maintained, and sections
prepared for histologic analysis. Intact cells were noted
within the confines of the artificial membranes and, as a
consequence, these formulations rendered viable cells for
implantation studies.

20

Example 3

In Vivo Studies of Encapsulated Cell Lines

Implanted in Nude Mice

 Cell viability following encapsulation was
25 evaluated in vivo using FL cells, a transformed line of
human amnion cells capable of tumor formation in the nude
mouse. The rationale for these experiments follows.
Cells encapsulated in alginate and implanted beneath the
skin of the nude mouse formed tumors as rapidly as
30 nonencapsulated cells injected subcutaneously, since the
alginate is rapidly dissolved in vivo. Formation of
tumors by cells encapsulated in polylysine was delayed,
since polylysine is not readily dissolved in the host and
cells first have to multiply within the capsules in
35 sufficient mass to burst them.

-18-

1 Encapsulated FL cells (Runs 1-15B, 1-16B, 1-36A,
and 1-36B) were maintained overnight at 37°C in an
atmosphere of O₂/CO₂(95/5 v/v). The following morning 0.5
ml alginate or polylysine encapsulated cells were
5 surgically implanted beneath the skin of 3-week old nude
mice of the nu/nu strain (Harlan). The mice were
sacrificed at 16 and 32 days after implantation for gross
and histological evaluation of tumor formation.

10 FL cell lines encapsulated in alginate and
implanted for a period of 16 days, demonstrated at
necropsy, viable cells with histologic features remarkably
similar to those described in the in vitro experiments
with the exceptions that the cell clusters were now much
larger, often forming confluent nests in excess of several
15 hundred cells.

Alginate membranes, as expected, were not visible
but the general outlines of the artificial cells were
present in some areas, perhaps attributable to
fibrocollagenous connective tissue proliferating in
20 proximity with the artificial cell membranes. In other
areas, FL cells had grown into confluent nests with the
subcutaneous tissue and muscles, violating and disrupting
the boundaries of the artificial cell membranes. In these
areas of host tumor interface, conspicuous bone and
25 osteoid production was noted.

FL cells encapsulated in polylysine and implanted
for 16 days again showed large viable cell clusters with
morphologic features as described with the exception that
the cell membranes of polylysine remained intact. Most
30 cell groups within the membranes had grown to confluency.
No evidence of cell penetration into adjacent tissues, as
was noted above, was apparent. No bone or osteoid
production was visible.

FL cell lines encapsulated in alginate/polylysine
35 harvested 32 days after implantation showed a large bulk

-19-

1 of tumor (larger than 2.0 X 1.0 cm) with FL cell line
morphology. It was composed of confluent nests and sheets
of cells proliferating in no discernable pattern with a
few groups similar to those described above still
5 present. Most membrane material apparently had been
resorbed and was inconspicuous. There was overt invasion
of host tissue by the FL cell lines with conspicuous bone
and osteoid production.

Thus, the above results have demonstrated the
10 viability of encapsulated cells and further that this
viability could be maintained throughout the implantation
or injection procedure with the encapsulated cells
subsequently proliferating within artificial membranes,
rupturing the membranes and invading into host tissues.

15 Additionally, the above results demonstrate that
cell lines induced bone production, evidence of the
maintenance of cell capacity to exhibit their normal
function following the encapsulation process. Alginate
and polylysine microcapsules apparently degrade at
20 different rates, since discernable differences between
polylysine and alginate encapsulated cells were noted at
16 days with alginate tending to degrade earlier than
polylysine.

In order to determine if artificially
25 encapsulated cells would survive in vivo, 7 nude mice were
injected with encapsulated cells formulated in varying
matrices. If injection of encapsulated cells is delayed,
viability is significantly suppressed. Vital cells
encapsulated in polylysine and alginate membranes could be
30 observed 24 days following injection. Surviving cells
which had been injected alone or in a carrageenan matrix
were not detectable at 24 days.

-20-

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Example 4

In vivo Studies of Treatment of Fracture Non-unions
Produced in Dogs.

Fracture nonunions were experimentally induced in
11 research grade dogs. The nonunions were performed by
surgically removing a 3 mm disc of cortical and cancellous
bone from the mid-radius. Dogs were then allowed to
resume normal weight bearing activities, and after 12
weeks, stable fracture nonunions were produced. The dogs
were then divided into groups consisting of controls
receiving only matrix material with no osteoprogenitor
cells and four animals receiving osteoprogenitor cells
formatted in varying ways. Each dog received cells which
had been harvested at the time of the initial surgery and
maintained in tissue culture as described in Example 1
above.

In order to facilitate handling during the
implantation procedures and to insure retention at the
nonunion site, the encapsulated cells were prepared in a
gel of low melt agarose (Sigma TYPE VII). A "doughnut"
prepared with 3 ml of 4% agarose was formed in a 28 mm
diameter culture dish with a 12 mm diameter post in the
center. After the agarose had gelled, the centerpost was
removed. A suspension was prepared from 3 ml encapsulated
cells and 3ml 2% agarose. The hole in the center of the
4% agarose doughnut was filled with 1.5 ml of this
suspension. After the central portion had gelled, the
entire doughnut was transferred to a cell culture dish,
covered with DMEM, and returned to the incubator. The
doughnuts were implanted into the nonunion defects within
15-18 hours. The outer rim of the doughnut was
substantial enough to permit gentle handling with
forceps. The central core was rigid enough to hold the
encapsulated cells at the implant site, while still
allowing for diffusion of wound and tissue fluid to the

-21-

1 cells. These discs were then implanted following
excision of the fibrous nonunion material and the radii
splinted with a 4-hole stainless steel splint. The dogs
then resumed weight bearing activity for an additional 12
5 weeks at which time the animal was sacrificed and material
taken for detailed histologic evaluation.

The two dogs receiving only polylysine matrix
material showed a persistent nonunion defect occupying
approximately 8.5-11% of the original nonunion defect
10 volume on histomorphometric analysis. The trabecular bone
volume in these areas was calculated at 6.5 and 24.75%
respectively with 46.9 and 18.9% fibrous connective tissue
intermixed as well as a small amount of fibrocartilage.
In addition, a significant quantity of polylysine matrix,
15 visible as irregularly shaped refractile material was
noted throughout the defect. There was a modest
multinucleate foreign body giant cell response to this
material as well as minimal chronic inflammatory cell
infiltration. The histologic features from the two
20 control dog studies were identical to six control dogs
from previous studies involving the encapsulation and
implantation of bone inductive proteins in nonunion
fractures.

When autologous osteoprogenitor cells were
25 encapsulated in an artificial matrix of alginate and
implanted in a dog nonunion, histologic examination showed
a dramatic and complete healing of the fracture nonunion.
This was apparent on histomorphometric analysis with 100%
of the original defect being filled with new bone. The
30 trabecular bone volume in this area was 55% with no
interposed fibrous connective tissue. Relatively normal
cancellous space was present instead. This was in
dramatic contrast to the controls and other test animals
receiving inductive proteins. Also apparent were isolated
35 small cell clusters and groups of cells with round,

-22-

1 elliptically located nuclei and relatively distinct
cytoplasmic membranes with slight eosinophilia to the
cytoplasm. These were identical to cell clusters noted in
the in vitro and nude mouse in vivo experiments. These
5 cells could be observed within the cancellous space and at
times in intimate adaptation with an acellular
eosinophilic homogeneous material consistent with osteoid.

When autologous osteoprogenitor cells were
encapsulated in a polylysine matrix and implanted in a dog
10 nonunion, histologic examination 3 weeks demonstrated
evidence of degrading artificial cell membranes consistent
with polylysine and a few artificial cell nests as
described above in the in vitro and in vivo nude mouse
studies, as well as the dog previously described.

15 Throughout the nonunion site there was evidence of brisk
osteoblastic activity with production of homogeneous,
eosinophilic acellular osteoid as noted in the previous
dog. The histologic features demonstrated healing at a
significantly advanced stage compared with that
20 anticipated for control animals from previous nonunion
experiments. The two remaining dogs each received
polylysine encapsulated cells or alginate encapsulated
cells. Both dogs were carried to 13 weeks. The
polylysine cells showed some evidence of osteoid
25 production and remnants of artificial cells, but no
significant fill of the nonunion defect. The same was
true of the last dog receiving alginate encapsulated cells.

The results of the implantation of
osteoprogenitor cells encapsulated in alginate (with or
30 without poly-L-lysine) demonstrated that the method of the
present invention causes complete healing of the fibrous
nonunion, the healed fracture being composed of mature
bone with lamellar characteristics and evidence that
remodeling of the fracture site into a functional state
35 had occurred. This Example conclusively demonstrates that

-23-

1 osteoprogenitor cells may be encapsulated in artificial
membranes, their viability maintained, and these cells
subsequently implanted in living subjects (mice and
5 dogs). The cells subsequently proliferate out of the
artificial confines to produce osteoid and new bone which
contributes to the healing process.

Although all dogs receiving encapsulated
osteoprogenitor cells did not demonstrate the same amount
of nonunion fracture healing, this result may relate to a
10 number of complex interrelated factors. These include the
kinetics of artificial cell membrane degradation, cell
release from artificial membranes, proliferative
capabilities of individual autologous cells, differences
inherent in healing capacity of each animal, or
15 combinations of these.

In addition, bone inductive factors may be
necessary in the artificial membranes to completely signal
encapsulated cell populations to begin proliferation
within the unfavorable environment of a healing wound.
20 Some of these variables may be overcome by inclusion of
bone cell differentiation factors within the microcapsule
at the time of encapsulation.

Example 5

Device Fixation Enhancement in Primates

25 In order to improve the fixation of titanium bone
implants, in vivo studies of implantation of
microencapsules containing osteoprogenitor cells and bone
inductive factors were performed. Osteoprogenitor cells
were encapsulated in either alginate or alginate coated
30 with polylysine as described in Example 1. Additionally,
microcapsules were prepared as in Example 1 however, bone
inductive growth factor was also included in the
microcapsules. The microcapsules were implanted within
the internal aspects of a titanium bone implant in six
35 sites in each of two baboons (animal no. 713 and 609) to

-24-

1 determine whether bone growth into titanium prosthetic
implants could be enhanced, facilitating of the fixation
of the prothesis in the baboon tibia.

5 One of six titanium implant sites was used as a
control and received no microcapsule material. Each of of
the remaining five titanium implant sites in baboon tibia
recieved the same microencapsulation composition. At one
week intervals for six weeks, tissue within the internal
10 aspects of one of the six titanium implant sites in each
baboon was retrieved for histologic evaluation. The
status of the encapsulated material and the quantity of
bone in the internal aspects of the titanium implant was
determined. Histologic analysis of tissue within the
15 implant at the site of microcapsule implantation was
carried out weekly for six weeks. The encapsulating
materials were highly biocompatible and did not elicit a
giant cell foreign body response. The amount of
encapsulating material present in histologic sections
decreased as the treatment period progressed, indicating
20 that the implanted microcapsules were biodegraded at the
site of the titanium prosthetic implant. Gross
histological examination revealed bone regeneration in all
titanium prosthetic implant sites which received the
microcapsule composition of the present invention.
25 Further analysis may reveal quantitative or qualitative
differences in the regenerating hard tissue due to the
presence of the bone inductive growth factor.

The invention now being fully described, it will
be apparent to one of ordinary skill in the art that many
30 changes and modifications can be made thereto without
departing from the spirit or scope of the invention as set
forth below.

WHAT IS CLAIMED AS NEW AND IS DESIRED TO BE
35 COVERED UNDER LETTERS PATENT IS:

-25-

CLAIMS

1. A composition comprising osteoprogenitor cells encapsulated in a biocompatible microcapsule.
2. The composition of claim 1, wherein said microcapsule comprises a biodegradeable polymer.
3. The composition of claim 2, wherein said biodegradeable polymer is selected from the group consisting of alginate, polylysine, methylcellulose, collagen, gellan gum, casein and chitosan.
4. The composition of claim 3 wherein said polymer is alginate.
5. The composition of claim 3 wherein said polymer is polylysine.
6. The composition of claim 1 wherein said microcapsule comprises alginate and polylysine.
7. The composition of claim 1 wherein said microcapsule comprises alginate coated with polylysine.
8. The composition of claim 1 further comprising a material selected from the group consisting of extracellular matrix of chondrocytes (ECM), a hormone, a growth factor, an antiviral agent, and an antibacterial agent.
9. The composition of claim 8 wherein said growth factor is selected from the group consisting of somatomedin, fibroblast growth factor, epidermal growth factor and bone derived growth factor.
10. The composition of claim 1 contained in a hydrogel wafer.
11. The composition of claim 10 wherein said hydrogel wafer comprises a material selected from the group consisting of agar, gelatin, gellan gum and agarose.
12. A method for promoting bone regeneration, comprising administration of the composition of any one of claims 1 - 11, inclusive, to an individual in need of said treatment.

-26-

1 13. The method of claim 12 wherein said individual is a mammal.

 14. The method of claim 13 wherein said mammal is selected from the group consisting of a human, a dog, a
5 cat, and a horse.

 15. The method of claim 14 where in said mammal is a human.

 16. A composition comprising a material resistant to degradation in biological fluids, wherein
10 said resistant material is selected from the group consisting of titanium oxide, hydroxyapatite, biocompatible metal compositions and biocompatible ceramic compositions; hard tissue promoting factors bound to the resistant material; and contained within a readily
15 degradeable polymer.

 17. The composition of claim 16 additionally comprising unbound hard tissue promoting factors.

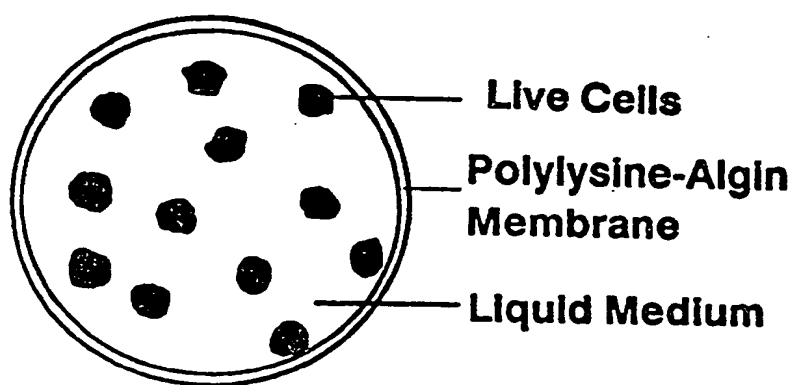
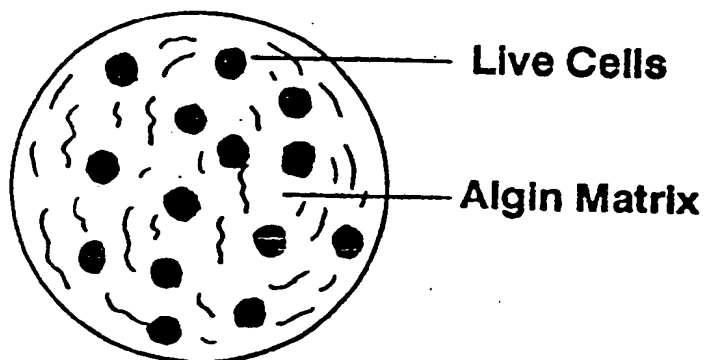
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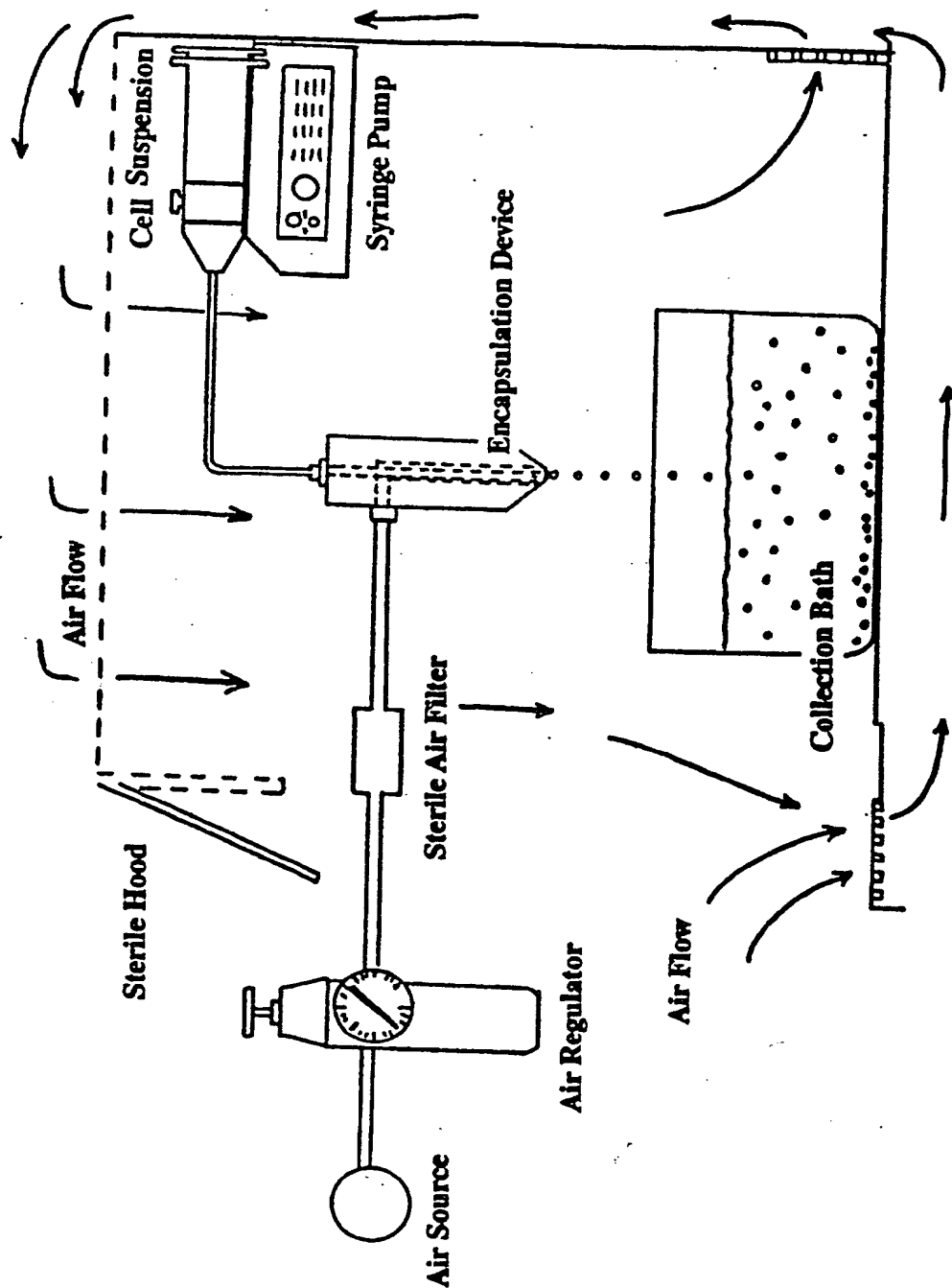
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TWO TYPES OF ARTIFICIAL CELLS

Figure 1

2/2



CELL ENCAPSULATION PROCESS

Figure 2

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/04381

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC (5): A61K 9/14, 9/16, 9/52, 9/64, 37/24; C12N 11/04, 11/10, 11/12
U.S. CL. 424/484, 485, 488, 491; 435/178, 182; 530/399

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	424/484, 485, 488, 491; 435/178, 179, 182 530/399

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A, P	US, A, 4,904,259 (ITAY) 27 FEBRUARY 1990 See Abstract.	1, 2, 12-15
Y	US, A, 4,642,120 (NEVO) 10 FEBRUARY 1987 See Abstract and column 4, lines 35-36.	1, 2, 8, 9, 12-15
Y	US, A, 4,609,551 (CAPLAN) 02 SEPTEMBER 1986 See Abstract, column 2, lines 1-2, 17-22, 54 and column 3, lines 34-48.	1-3, 8, 12-15
Y	US, A, 4,647,536 (MOSBACH) 03 MARCH 1987 See Abstract and column 1, lines 10-15.	1, 2, 3
Y	US, A, 4,663,286 (TSANG) 05 MAY 1987 See Abstract, column 1, lines 5-7 and column 3, lines 4-38.	1-7
Y	US, A, 4,391,909 (LIM) 05 JULY 1983 See Abstract, column 4, lines 9-16, column 9, and Example 1.	1-7
Y	US, A, 4,673,566 (GOOSEN) 16 JUNE 1987 See Abstract, column 8 and Example 1.	1-7, 10, 11

* Special categories of cited documents: ¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATE

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 OCTOBER 1990	09 JAN 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	S. Kishore Gollamudi S. Kishore

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4,798,786 (TICE) 17 JANUARY 1989 See Abstract, column 10 and claim 3.	1-3, 11-15
Y	US, A, 4,620,327 (CAPLAN) 04 NOVEMBER 1986 See Abstract, column 2, lines 4-54 and column 3, lines 27-34.	16 & 17
Y	US, A, 4,610,692 (EITENMULLER) 09 SEPTEMBER 1986; See Abstract.	16 & 17
Y	US, A, 4,595,713 (ST. JOHN) 17 JUNE 1986 See Abstract, and column 6, lines 23-41.	16 & 17
Y	US, A, 4,888,366 (CHU) 19 DECEMBER 1989 See Abstract, column 2, lines 54-64 and column 7, lines 7-65.	16 & 17

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹²not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-15: A composition comprising osteoprogenitor cells encapsulated in a microcapsule.
- II. Claims 16-17: A composition containing no osteoprogenitor cells.
(See attachment)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Con't. from PCT/ISA/210 supplemental sheet (2).

The international application lacks unity of invention under PCT Rule 13 because of the following reason:

Inventions I and II are independent and distinct in that the composition in invention I contains osteoprogenitor cells where as the composition in invention II does not require the presence of these cells, but contain additional factors.